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Visfatin increases the invasive potential of ovarian granulosa tumor spheroids by reprogramming glucose metabolism

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Abstract

In brief: The role of visfatin in ovarian granulosa cell tumor (GCT) invasion and glucose metabolism reprogramming is largely unexplored. These studies imply that visfatin or its inhibitor is involved in regulating ovarian granuloma invasion by reprogramming glucose metabolism and may be a potential candidate for the diagnosis and treatment of ovarian GCT.

Abstract: Visfatin is an adipokine with nicotinamide phosphoribosyltransferase (NAMPT) activity, the concentration of which is higher in ascitic fluid than in serum, and is associated with ovarian cancer peritoneal dissemination. Potentially important effects of visfatin on glucose metabolism have been previously reported. However, the mechanism underlying the effects of visfatin on ovarian cancer cell invasion, and whether this involves altered glucose metabolism, has not been elucidated. Here, we tested the hypothesis that visfatin, which can reprogram cancer metabolism, promotes invasion by ovarian cancer spheroids. Visfatin increased glucose transporter (GLUT)1 expression and glucose uptake in adult granulosa cell tumor-derived spheroid cells (KGN) and also increased the activities of hexokinase 2 and lactate dehydrogenase. We showed a visfatin-induced increase in glycolysis in KGN cells. Moreover, visfatin increased the potential invasiveness of KGN spheroid cells by upregulating *MMP2* (matrix metalloproteinase 2) and downregulating *CLDN3* and *CLDN4* (claudin 3 and 4) gene expression. Interestingly, an inhibitor of GLUT1 and lactate dehydrogenase (LDHA) abolished the stimulatory effect of visfatin on the potential invasiveness of KGN cells. More importantly, silencing expression of the *NAMPT* gene in KGN cells demonstrated its important effect on glycolysis and invasiveness in adult granulosa cell tumor cells (AGCTs). In summary, visfatin appears to increase AGCT invasiveness through effects on glucose metabolism and to be an important regulator of glucose metabolism in these cells.

Reproduction (2023) **165** 521–531

Introduction

Visfatin, also called nicotinamide phosphoribosyltransferase (NAMPT) or pre-B-cell colony-enhancing factor, is an adipocytokine and cytosolic enzyme with NAMPT activity that is principally produced by perivascular adipose tissue but also by the liver and immune cells (Luk *et al.* 2008, Garten *et al.* 2010). Visfatin regulates physiological processes such as cell proliferation and glucose metabolism and specifically increases glucose uptake (Saddi-Rosa *et al.* 2010, Liang *et al.* 2016).

It is well known that increases in glucose uptake and NADPH production contribute to the invasive phenotype in cancer cells (Cairns *et al.* 2011). The ability of cancer cells to increase their glucose uptake and switch from oxidative metabolism to glycolysis to produce lactate even when oxygen is abundant (defined as the 'Warburg effect') promotes invasiveness and metastasis (Doherty & Cleveland 2013). Furthermore, lactic acid is an inducer of epithelial–mesenchymal transition (EMT), which occurs in response to the consequent reduction in the pH of the cancer environment (Damgaci *et al.* 2018). Visfatin reprograms the metabolism of metastatic melanoma (Audrito *et al.* 2020). However, the use of an inhibitor of NAMPT inhibits the progression of ovarian tumors (Piacente *et al.* 2017, Nacarelli *et al.* 2020). Recently, Kudo *et al.* (2020) reported that ovarian cancer cell lines can be divided into subgroups exhibiting NAMPT-dependent or NAMPT-independent glycolysis; however, the indirect role of visfatin in inducing invasiveness of ovarian cancer via the regulation of metabolic reprograming remains to be evaluated. Previous studies have assessed visfatin overexpression in ovarian cancer cells (Shackelford *et al.* 2010, Reverchon *et al.* 2013). Immunohistochemistry showed high visfatin expression in ovarian cancer cell and low expression in benign ovarian tissue (Shackelford *et al.* 2010). Moreover, Li *et al.* (2015) showed that the serum visfatin concentrations in patients with ovarian cancer were higher than those in controls (58.12 ± 33.9 ng/mL

ISSN 1470–1626 (paper) 1741–7899 (online)
https://doi.org/10.1530/REP-22-0443
Online version via https://rep.bioscientifica.com

vs 42.07 ± 21.1 ng/mL, respectively) and importantly that the concentrations of visfatin in ascitic fluid from patients with ovarian cancer were higher than the serum concentrations, strongly suggesting an association with peritoneal dissemination of ovarian cancer.

Clinical and case studies show that both epithelial and granulosa ovarian tumor cells, either as single cells or as spheroid-like structures, are present in the ascitic fluid from patients with ovarian cancer, and this is thought to favor peritoneal dissemination (Azad & Khunger 2010). Granulosa cell tumors (GCT) account for 3–5% of ovarian cancers. The most frequently diagnosed subtype of GCT is the adult granulosa cell tumor (AGCT) (Nishi *et al.* 2001), represented by KGN cell lines derived from patients with invasive ovarian GCT. Although GCTs are thought to have a better prognosis than epithelial tumors, AGCTs are characterized by frequent recurrence or metastasis 10–20 years after the removal of the primary tumor (Miller & McCluggage 2008). Furthermore, SKOV3 cells, an adenocarcinoma cell line with epithelial like morphology derived from the ascitic fluid of an ovarian cancer patient, are most likely to represent clear-cell cancer (Beaufort *et al.* 2014).

These findings led to the intriguing hypothesis that visfatin, which is present in the ascitic fluid of patients with ovarian cancer and can reprogram cancer metabolism, may promote invasiveness of ovarian cancers. Therefore, in the present study, we aimed to determine whether visfatin promotes the invasiveness of ovarian cancer spheroids by reprogramming their metabolism. To this end, a human epithelial ovarian cancer cell line obtained from ascitic fluid (SKOV-3) and a human AGCT-derived cell line (KGN) were used as *in vitro* models. Using these, we determined whether visfatin stimulates the invasiveness of ovarian cancer, evaluated the effects of visfatin on glucose metabolism in cancer cells, and explored the molecular and cellular mechanisms underlying these processes.

Materials and methods

Chemical treatments

Visfatin (NAMPT) (R&D Systems, Inc.) was used as the test compound and was dissolved in PBS (Thermo Fisher Scientific). A potent, noncompetitive NAMPT inhibitor (FK 866 hydrochloride) was purchased from R&D Systems, Inc., and a selective glucose transporter 1 (GLUT1) inhibitor (STF-31) and lactate dehydrogenase (LDHA) inhibitor (GSK2837808A) were purchased from Sigma-Aldrich; both were dissolved in dimethyl sulfoxide (DMSO). The final concentration of DMSO in the media was <0.1% (v/v). Mitomycin C was purchased from Sigma-Aldrich and dissolved in cell culture water (Thermo Fisher Scientific).

Cell culture

The human epithelial ovarian cancer cell line SKOV-3 (ATCC), which was originally derived from the ascitic fluid, and the human AGCT-derived cell line KGN (Riken Cell Bank (RBRC-RCB1154), Ibaraki, Japan), the supply of which was approved by Drs Yoshiro Nishi and Toshihiko Yanase, were used as *in vitro* model systems. KGN cells were cultured in DMEM/Ham's F12 medium without phenol red (Thermo Fisher Scientific) supplemented with 10% charcoal-stripped fetal bovine serum (FBS; Biowest, Nuaillé, France). SKOV-3 cells were maintained in McCoy's 5A medium (Sigma-Aldrich) supplemented with 10% FBS. The cells were incubated at 37°C in a 95% air/5% CO_2-containing humidified incubator.

Spheroid formation

To form spheroids, SKOV-3 and KGN cells (5000 and 9000 cells per spheroid, respectively) were transferred to media containing 10% FBS and methylcellulose solution (Sigma-Aldrich) at a final concentration of 0.25%. The cells were seeded in 96-well CellStar U-bottomed plates (Greiner Bio-One, Kremsmünster, Austria) and centrifuged for 10 min. Then, the cells were incubated for 72 h to allow spheroid formation.

Cell treatments

KGN and SKOV-3 spheroids were exposed to visfatin at concentrations of 10, 100, or 1000 ng/mL for 24 h, and then their invasive potential, glucose uptake, gene expression, and HK2 and LDH activities were assessed. After 48 h, their invasive potential and protein expression were also assessed. To investigate the role of visfatin in spheroid invasiveness, the cells were pretreated for 1 h with an NAMPT inhibitor (FK866; 1 nM) before being exposed to visfatin. The involvement of glycolysis in the effects of the visfatin was investigated by pretreating cells with an inhibitor of GLUT1 (STF-31, 2 μM) or with an LDHA inhibitor (GSK2837808A, 20 μM) for 1 h, prior to exposure to visfatin.

Glucose uptake

KGN and SKOV-3 spheroid cells were treated with visfatin (10, 100, or 1000 ng/mL) for 24 h. After the removal of the culture medium, cells were washed with PBS and then incubated with 1 mM 2-deoxyglucose (2-DG) for 10 min at room temperature. The uptake of the 2-DG was stopped by the addition of an acid detergent solution and neutralization using a neutralization buffer. 2-Deoxyglucose-6-phosphate (2-DG6P) detection reagent was then added, and the cells were incubated for 1 h, after which luciferase activity was measured using a Glucose Uptake-Glo Assay (Promega, Madison, WI, USA), according to the manufacturer's instructions, and a SpectraMax L luminometer (Molecular Devices, San Jose, CA, USA).

Analysis of 3D spheroid invasiveness

KGN and SKOV-3 spheroids were pretreated (or not) with visfatin (100 ng/mL) and incubated at 37°C in a 95% air/5% CO_2 humidified incubator. Next, 50 μL Cultrex® Spheroid Invasion Matrix (#3500-096-03; Trevigen Inc., Gaithersburg, MD, USA) were added to each well of a 3D culture 96-well spheroid formation plate. The plate was centrifuged at 300 ***g***

for 5 min and then incubated at 37°C for 1 h to promote gel formation. Afterward, 100 µL complete medium containing visfatin (100 ng/mL) were added to each well, and the cells were incubated at 37°C for 24 h. Invasion by the spheroids was visualized using a bright-field microscope (Axiocam 503; 5× objective; Zeiss, Oberkochen, Germany) after 0, 24, and 48 h. The area of invasion was measured using ImageJ software using thresholding feature (National Institutes of Health) according to the manufacturer's instructions (Cultrex® 96-Well 3D Spheroid BME Cell Invasion Assay Protocol). Resulting invasion areas were divided by the area obtained for the control, providing the invasion index as a percentage of control.

Real-time PCR

RNA isolation and cDNA synthesis were carried out on spheroids at baseline and after treatment with visfatin (100 ng/mL) for 24 h, using the TaqMan Gene Expression Cells-to-CT kit (Applied Biosystems/ThermoFisher Scientific) according to the manufacturer's instructions. The lysis solution contained DNase I to remove genomic DNA contamination. The resulting pre-amplified cDNA preparations were analyzed by real-time PCR using the StepOne-Plus real-time PCR system (Applied Biosystems/ThermoFisher Scientific) and TaqMan gene expression assays in combination with TaqMan gene expression master mix containing the ROX passive reference dye (Applied Biosystems/ThermoFisher Scientific). The thermal cycling conditions were as follows: 50°C for 2 min, 95°C for 10 min, and then 40 cycles of 95°C for 15 s and 60°C for 60 s. Duplicate control samples lacking cDNA were prepared for each gene and showed no amplification. The following TaqMan gene expression assays were used: metalloproteinases 2 and 9 (*MMP2*; Hs01548727_m1; *MMP9*; Hs00957562_m1); E-cadherin (*CDH1*; Hs01023894_m1); N-cadherin (*CDH2*; Hs00983056_m1); claudins 1, 3, and 4 (*CLDN1*; Hs00221623_m1; *CLDN3*; Hs00265816_s1; *CLDN4*; Hs00976831_s1); GLUT1 (*SLC2A1*; Hs00892681_m1); hexokinase 2 (*HK2*; Hs00606086_m1); and *LDHA* (Hs00855332_g1). The relative gene expression levels were normalized to that of *GAPDH* (4310884E) and were calculated using the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen 2001).

Western blot analysis

After treating spheroids with visfatin (100 ng/mL) for 48 h, spheroids were lysed in lysis buffer. Proteins were separated on 4–20% Mini-Protean TGX Precast Protein Gels (Bio-Rad) and then transferred to Trans-Blot Turbo Mini PVDF transfer packs (Bio-Rad) using the Trans-Blot Turbo transfer system (Bio-Rad). The blots were blocked for 1 h with 0.02 M Tris-buffered saline containing 5% bovine serum albumin and 0.1% Tween 20 and then incubated overnight at 4°C with antibodies specific for MMP2 (#4022; Cell Signaling Technology), GLUT1 (ab652; Abcam), GLUT4 (#2213; Cell Signaling Technology), hypoxia-inducible factor (HIF-1; #41493; GeneTex), and visfatin (NAMPT; ab233294; Abcam). The membranes were then washed three times in Tris-buffered saline and 0.1% Tween 20 and incubated for 1 h at room temperature with horseradish peroxidase (HRP)-conjugated anti-rabbit (#7074) or anti-mouse (#7076) secondary antibodies (Cell Signaling Technology). β-actin (A5316; Sigma-Aldrich) was used as a loading control. Immunopositive bands were visualized using WesternBright Sirius HRP substrate (Advansta, Menlo Park, CA, USA). Quantification of protein bands from three independent experiments was performed by densitometry using VisionWorks LS Acquisition and Analysis software (UVP, Upland, CA, USA).

Measurement of hexokinase 2 and lactate dehydrogenase activities

The HK2 and LDHA activities of KGN spheroid cells were measured after 24 h of treatment with visfatin (100 ng/mL) using a hexokinase colorimetric assay kit (Sigma-Aldrich; cat. number: MAK091) and a lactate dehydrogenase activity assay kit (Sigma-Aldrich; cat. number: MAK066), respectively, according to the manufacturer's instructions.

Analysis of glucose metabolism

The extracellular acidification rate (ECAR) and oxygen consumption rate (OCR) of cells were measured using an XFp Extracellular Flux analyzer (Seahorse Bioscience, North Billerica, MA, USA) and an XFp Glycolytic Rate Assay Kit (Seahorse Bioscience), according to the manufacturer's instructions. KGN cells were seeded at a density of 10,000 cells/well in an XFp cell culture microplate in phenol red-free DMEM/Ham's F12 (Thermo Fisher) supplemented with 10% FBS (Biowest). The cells were treated for 24 h with visfatin (100 ng/mL). Prior to the start of the assay, the media were removed, and the cells were washed with nonbuffered Seahorse XF DMEM assay buffer pH 7.4 supplemented with 2 mM glutamine, 1 mM pyruvate, and 10 mM glucose. Approximately 180 µL of the XF assay medium were added to each well, and the cells were incubated in a CO_2-free incubator for 1 h. ECAR and OCR measurements were made after the addition of visfatin (100 ng/mL), followed by rotenone and antimycin A (Rot/AA (0.5 µM)) and finally 2-DG (50 mM). XFp assays were performed on three occasions in triplicate. The data were analyzed using Seahorse Wave software (Seahorse Bioscience) and then exported to Prism 8 (GraphPad) for statistical analysis.

NAMPT gene silencing

Small-interfering RNAs (siRNAs) were introduced into KGN cells using DharmaFECT 3 Transfection Reagent (GE Healthcare Dharmacon Inc.), according to the manufacturer's instructions, to silence NAMPT. Briefly, spheroids were cultured in serum-free medium containing DharmaFECT 3 Transfection Reagent containing nontargeting (control) siRNA or targeting siRNA at a final concentration of 100 nM (SMARTpool siRNA, a mixture of four siRNAs provided as a single reagent targeting human NAMPT (SMART pool siRNA NAMPT; L-004581-00-0005)) for 24 h. The gene knockdown efficiency was evaluated 24 h after siRNA transfection by real-time PCR as well as 48 h and 72 h by western blot. Basal glycolysis and invasiveness after siNAMPT transfection were assessed using a Seahorse Glycolytic Rate

Assay (Agilent Technologies) and Cultrex® Spheroid Invasion Matrix (#3500-096-03; Trevigen Inc.), respectively.

Statistical analysis

Statistical analysis was performed using Prism software (GraphPad). The mean ± S.E.M. values of datasets generated in three independent experiments, performed in triplicate, were compared using one-way ANOVA, followed by Tukey's test or Student's *t*-tests (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

Results

Visfatin increases glucose uptake by KGN but not SKOV-3 spheroids

First, we determined whether visfatin altered glucose uptake by the human cancer cell lines KGN and SKOV-3, as previously reported for other cell models. Treatment with 10, 100, or 1000 ng/mL visfatin significantly increased glucose uptake by KGN cells (by 1.41-, 1.35-, and 1.22-fold, respectively) (Fig. 1A, $P < 0.01$ or $P < 0.001$). However, it did not affect glucose uptake by SKOV-3 cells (Fig. 1B). To determine whether GLUT1 was involved in this effect of visfatin, we first measured the expression of *GLUT1* in the SKOV-3 and KGN cells and found that it was significantly higher (1.78-fold) in KGN cells than in SKOV-3 cells (Fig. 1C, $P < 0.01$). Next, we determined whether visfatin at a concentration of 100 ng/mL would affect the protein expression of GLUT1 and GLUT4 in KGN cells and found that it increased the expression of GLUT1 protein but not that of GLUT4 protein (Fig. 1D and E, $P < 0.001$).

Glycolytic enzymes are activated by visfatin in KGN cells

After transport into cells, glucose is phosphorylated by HK2, and the final product of glycolysis is lactate, which is formed by the reduction of pyruvate, catalyzed by LDHA. Therefore, we measured the mRNA expression of the genes encoding these enzymes in SKOV-3 and KGN cells. The KGN cells showed significantly higher *HK2* mRNA expression (7.36-fold) than the SKOV-3 cells, but there was no difference in the mRNA expression of *LDHA* (Fig. 2A and D; $P < 0.001$). Next, we determined whether visfatin treatment altered the expression or activity of HK2 or LDH in KGN cells, respectively, and found that it significantly increased HK2 activity by 1.27-fold (Fig. 2C; $P < 0.01$) but had no effect on *HK2* mRNA expression (Fig. 2B). In addition, treatment with visfatin increased the *LDHA* mRNA expression and the activity of LDH by 1.12-fold and 1.26-fold, respectively (Fig. 2E and F; $P < 0.05$ and $P < 0.001$, respectively).

Visfatin increases the invasive potential of KGN but not SKOV-3 cell spheroids

Cancer invasiveness is associated with a change in glucose metabolism. Therefore, we evaluated the invasive potential of KGN and SKOV-3 spheroids after treatment with 100 ng/mL of visfatin (NAMPT) for 24 h or 48 h using a Spheroid BME Cell Invasion Assay. We found that visfatin stimulated the invasiveness of KGN spheroids by 1.26- and 1.51-fold vs control, respectively (Fig. 3A, $P < 0.001$). In addition, pretreatment of KGN cells with the visfatin inhibitor FK866 for 1 h reduced the stimulatory

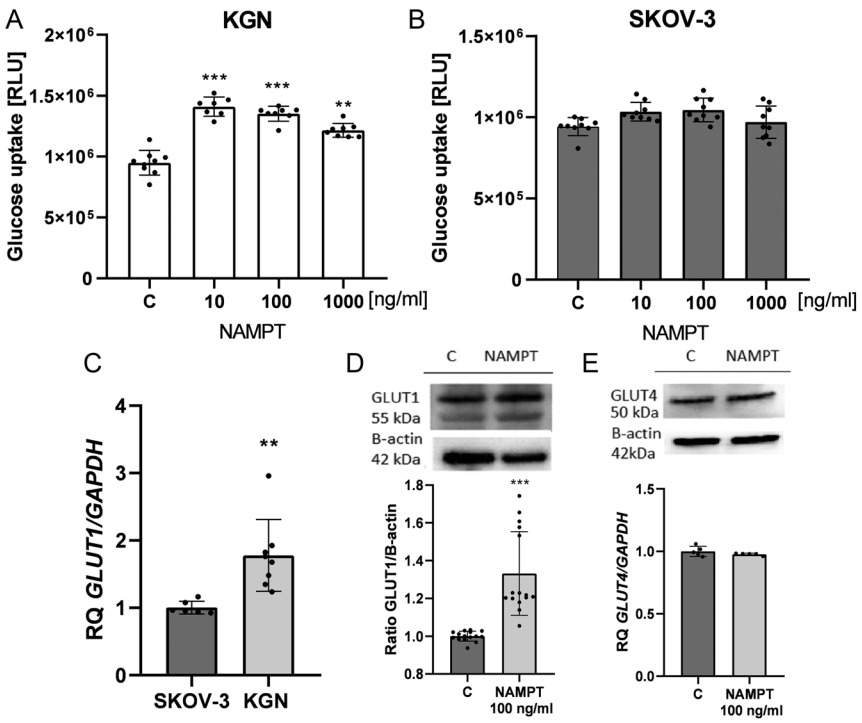


Figure 1 Effect of treatment with visfatin (NAMPT) (100 ng/mL) for 48 h on glucose uptake by KGN (A) and SKOV-3 (B) cells. Basal mRNA expression of GLUT1 in SKOV-3 and KGN cells (C). Effect of visfatin (100 ng/mL) treatment for 48 h on the protein expression of GLUT1 (D) and GLUT4 (E) in KGN cells. RLU, relative luminescence units. Data are mean ± S.E.M. of three independent experiments. C, control. **$P < 0.01$, ***$P < 0.001$.

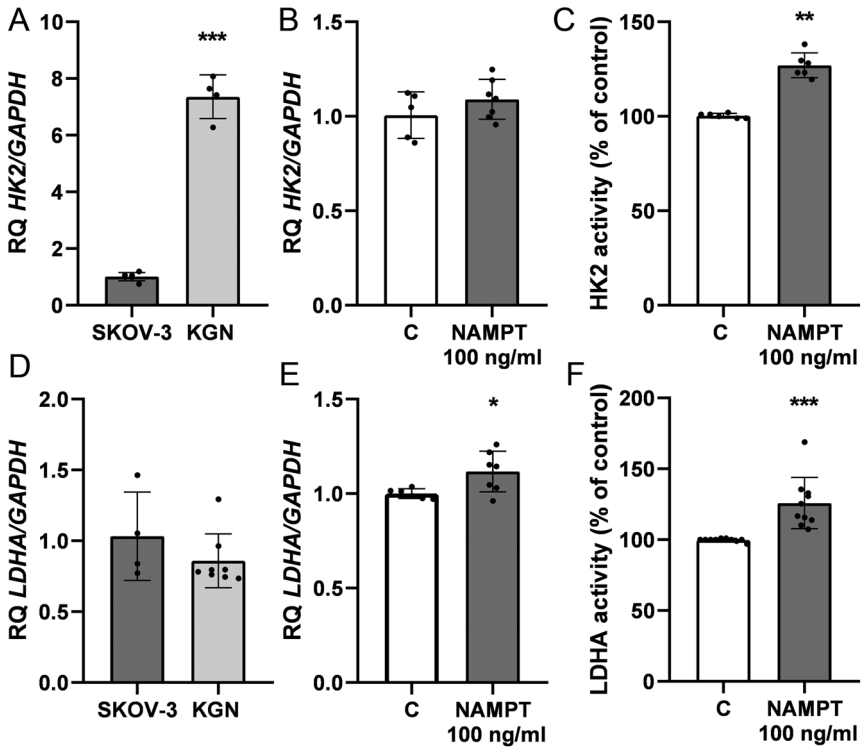


Figure 2 Basal mRNA expression of HK2 (A) and LDHA (D) in SKOV-3 and KGN cells. Effects of 24 h of treatment with visfatin (NAMPT) (100 ng/mL) on HK2 (B) and LDHA (E) mRNA expression, and HK2 (C) and LDH (F) activities in KGN cells. The mRNA expression in vehicle-treated cells was set to 1.0. RQ, relative quantity. Each bar represents the mean ± S.E.M. of three independent experiments. C, control. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

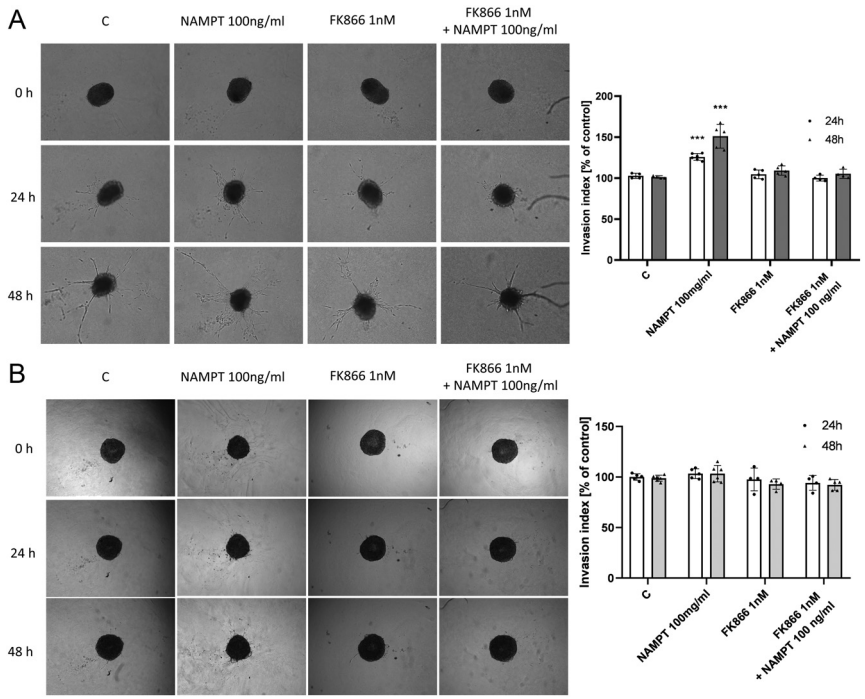


Figure 3 Effect of 24 h of treatment with visfatin (NAMPT) (100 ng/mL) and an inhibitor of NAMPT (FK866; 1nM) on the invasiveness of (A) KGN and (B) SKOV-3 cell spheroids. Data represent the mean ± S.E.M. of three independent experiments. C, control. $^{***}P < 0.001$.

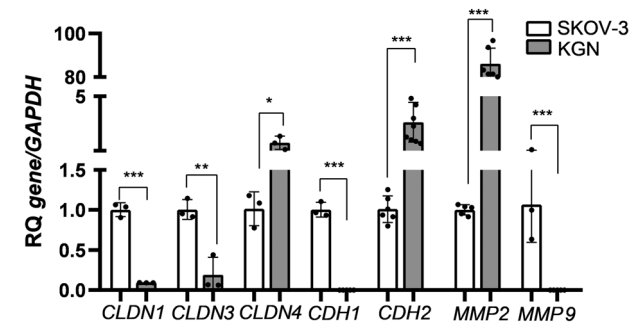
effect of visfatin on spheroid invasiveness to control levels (Fig. 3A). By contrast, treatment with visfatin did not affect the invasiveness of SKOV-3 cell spheroids (Fig. 3B).

SKOV-3 and KGN cell lines possess different basal invasion potentials

To identify the mechanisms underlying the difference in invasiveness between SKOV-3 and KGN cell lines, we compared the basal expression of genes encoding epithelial proteins (claudins 1, 3, and 4 (*CLDN1*, *CLDN3*, *CLDN4*) and E-cadherin (*CDH1*)) and mesenchymal proteins (N-cadherin (CDH2)) and matrix metalloproteinases 2 (*MMP2*) and 9 (*MMP9*)). In KGN cells, the basal expression of CLDN1 and CLDN3 was 10- and 5-fold lower, respectively, than in SKOV-3 cells. In KGN cells, *CDH1* and *MMP9* expression could not be detected, as reported previously in our studies, but both genes were expressed in SKOV-3 cells. Moreover, the expression of CDH2 was three-fold higher in KGN cells than in SKOV-3 cells, and the expression of MMP2 was 86-fold higher in KGN cells (Fig. 4, $P < 0.001$, $P < 0.01$, and $P < 0.05$, respectively). In summary, KGN cells show lower expression of epithelial proteins, such as claudins 1 and 3, and do not express E-cadherin, whereas they express higher levels of N-cadherin and metalloproteinase 2, implying that KGN cells have a more mesenchymal phenotype than SKOV-3 cells.

Visfatin increases MMP2 and reduces claudins 3 and 4 expression in KGN spheroids

To further explain the differences in the effects of visfatin on the invasiveness of SKOV-3 and KGN spheroids, we measured the expression of genes encoding epithelial and mesenchymal proteins in both cell sets of spheroids following exposure to visfatin. In KGN cells, visfatin (100 ng/mL) reduced the expression of *epithelial genes claudin CLDN3* (1.64-fold) and *CLDN4* (1.35-fold) and increased MMP2 expression at both the gene (1.31-fold) and protein (1.31-fold) levels (Fig. 5A, B, E and F; ***$P < 0.001$; **$P < 0.01$ *$P < 0.05$). However, visfatin did not affect the expression of *CLDN1*, *CLDN3*, *CLDN4*, *CDH1*, *CDH2*, or *MMP2* in SKOV-3 cells (Fig. 5G, H, I, J, K and L). These data are consistent with visfatin having no effect on SKOV-3 invasiveness.



	Basal EMT genes expression						
	epithelial genes				mesenchymal genes	metaloproteinases	
	CLDN1	CLDN3	CLDN4	CDH1	CDH2	MMP2	MMP9
SKOV-3	1.00 ± 0.09	1.00 ± 0.12	1.02 ± 0.21	1.0 ± 0.09	1.01 ± 0.16	1.0 ± 0.06	1.07 ± 0.47
KGN	0.09 ±0.001	0.19 ± 0.22	2.01 ± 0.42	ND	3.33 ± 1.28	86.1 ± 7.20	ND
P	***	**	*	***	***	***	***

Figure 4 Basal mRNA expression of epithelial–mesenchymal transition (EMT) genes in SKOV-3 and KGN spheroids. The level of mRNA expression in SKOV-3 cells was set to 1.0; RQ, relative quantity. Data represent the mean ± S.E.M. of three independent experiments *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ for SKOV-3 cells vs KGN cells. ND, not detected.

Visfatin increases HIF1 expression and glycolysis in KGN cell spheroids

HIF1 is a key mediator of the switch from aerobic metabolism to glycolysis, which is characteristic of cancer metabolism and essential for the invasiveness of tumor cells. We found that visfatin increased the expression of HIF1 protein (2.02-fold) (Fig. 6A, $P < 0.001$). Furthermore, we analyzed the bioenergetic profile of KGN cells using a Seahorse XFe96 Analyzer and found that visfatin treatment increased glycolysis. The basal and stimulated rates of glycolysis were significantly higher in KGN cells treated with visfatin for 24 h (55.8 vs 76.8 pmol/min and 61.2 vs 80.6 pmol/min, respectively) (Fig. 6B and C; $p < 0.05$). The baseline ECAR and proton efflux rate (PER) in response to NAMPT, Rot/AA, and 2-DG differed between control and visfatin-treated KGN cells (Fig. 6D and E). Taken together, these findings imply that visfatin stimulates glycolysis in KGN cells.

Silencing visfatin expression reduces the glycolytic rate and invasive potential of KGN spheroids

To further strengthen our findings, we next silenced the NAMPT expression using siRNA and assessed glycolysis in KGN cells. The transfection of KGN cells with siRNAs specific for NAMPT at doses of 50 or 100 nM reduced *NAMPT* expression by 62% and 49%, respectively (Fig. 7A; $P < 0.01$, $P < 0.001$). Therefore, in further analyses, we used 100 nM of siRNA. Moreover, visfatin expression at the protein level after siNAMPT transfection was 41% and 35% after 48 h and 72 h, respectively, compared to cells transfected siNT (Fig. 7B; $P < 0.001$). The transfection of KGN with siRNAs targeting NAMPT reduced the basal rate of glycolysis (Fig. 7C, $P < 0.05$). In addition, there were differences in ECAR and PER responses to Rot/AA and 2-DG between control and siRNA-treated KGN cells (Fig. 7D and E). Furthermore, silencing NAMPT decreased the invasive potential of KGN cells to 25.6 and 45.24% of that of non-silenced cells 24 h and 48 h, respectively (Fig. 7F; $P < 0.01$, $P < 0.001$). These findings are consistent with visfatin-mediated regulation of glycolysis in AGCTs.

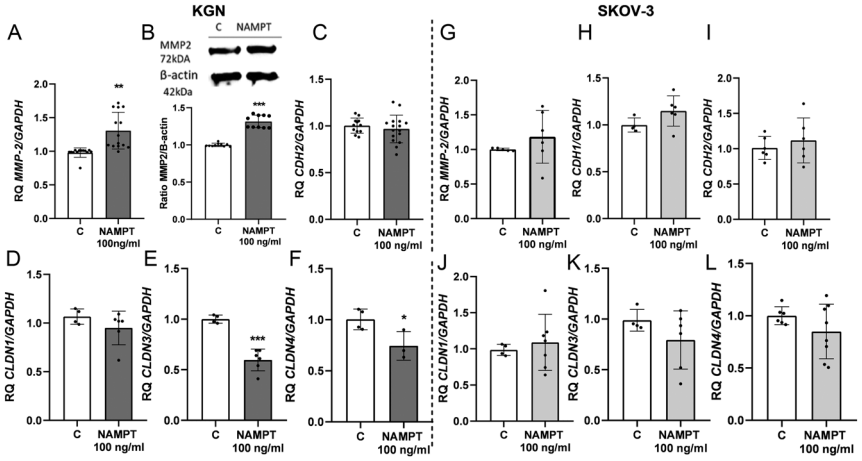


Figure 5 Effects of visfatin (NAMPT) (100 ng/mL) on the expression of matrix metalloproteinase 2 (MMP2) mRNA (A) and protein (B) and the mRNA expression of N-cadherin (CDH2) (C), claudin 1 (CLDN1) (D), claudin 3 (CLDN3) (E), and claudin 4 (CLDN4) (F) in KGN cells. mRNA expression and MMP2 protein expression were measured after 24 h and 48 h, respectively. Effects of visfatin (100 ng/mL) treatment for 24 h on the mRNA expression of MMP2 (G), E-cadherin (CDH1) (H), CDH2 (I), CLDN1 (J), CLDN3 (K), and CLDN4 (L) in SKOV-3 cells. The expression of each mRNA in vehicle-treated cells was set to 1.0. RQ, relative quantity. Data represent the mean ± S.E.M. of three independent experiments. C, control. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

Visfatin increases the invasiveness of KGN spheroids through its effect on glycolysis

Finally, we used an inhibitor of GLUT1 (STF-31, 2 μM) and an inhibitor of LDHA (GSK2837808A) to determine whether glucose availability and blockade of lactate dehydrogenase directly affect the invasiveness of KGN spheroid cells. KGN cells were pretreated for 1 h with STF-31 or GSK2837808A and then exposed to visfatin (100 ng/mL) for 24 h. STF-31 treatment reduced the invasiveness of KGN cells, and simultaneous treatment with visfatin did not abrogate this effect (Fig. 8A; $P < 0.001$). Similarly, GSK2837808A decreased the invasiveness of KGN cells (Fig. 8B, $P < 0.05$, $P < 0.001$). This implies that glucose availability and glycolysis are important for the invasiveness of AGCT spheroids.

Discussion

Previous studies show that visfatin is present in the ascitic fluid from patients with ovarian cancer (75.3 ± 28.1 ng/mL) and that it affects the intraperitoneal dissemination of ovarian cancer (Li *et al.* 2015) and glucose metabolism (Xie *et al.* 2007, Saddi-Rosa *et al.* 2010). It is well known that tumor cells, including ovarian cancer cells, obtain energy in a characteristic way (Nantasupha *et al.* 2021). The most characteristic feature of tumor metabolism is the 'Warburg effect', which is a shift in the source of ATP from oxidative phosphorylation to glycolysis, even under normal oxygen tension (Warburg 1956). However, the relationship between these two effects of visfatin in ovarian cancer has remained unclear and may have important health implications. Because visfatin is found in the ascitic fluid, we analyzed its effects on different types of ovarian cancer cell lines: an epithelial ovarian cancer originally obtained from the ascitic fluid (SKOV-3) (Domcke *et al.* 2013) and a human GCT-derived cell line (KGN) (Nishi *et al.* 2001).

Several studies show that visfatin treatment induces the migration of ovarian epithelial cancer cells (Caov-3 cells) (Li *et al.* 2015) and promotes the progression

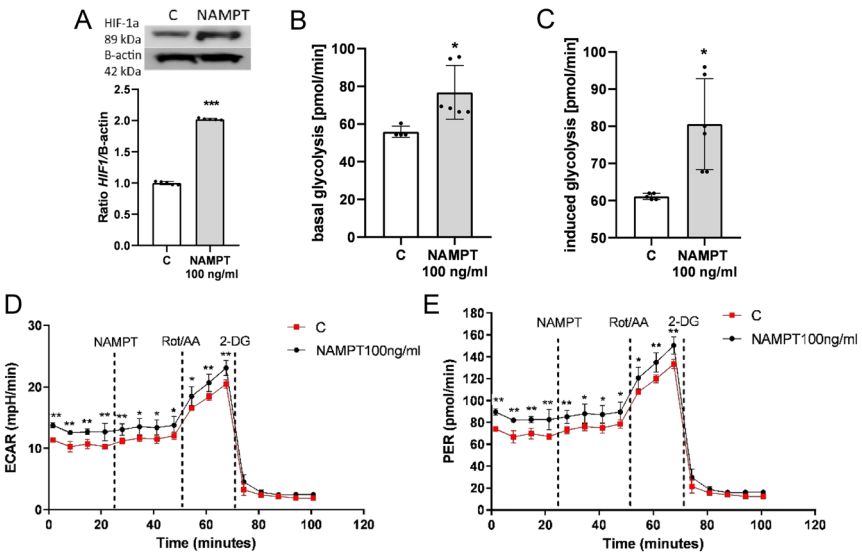


Figure 6 Effect of visfatin (NAMPT) treatment for 24 h on the protein (A) expression of HIF1 after 48 h and basal glycolysis (B) or stimulated glycolysis (C) in KGN cells. Representative ECAR (D) and PER (E) profiles of KGN cells following NAMPT, Rot/AA, and 2-DG treatment. Data represent the mean ± S.E.M. of three independent experiments. C, control. $^{*}P < 0.05$, $^{**}P < 0.01$.

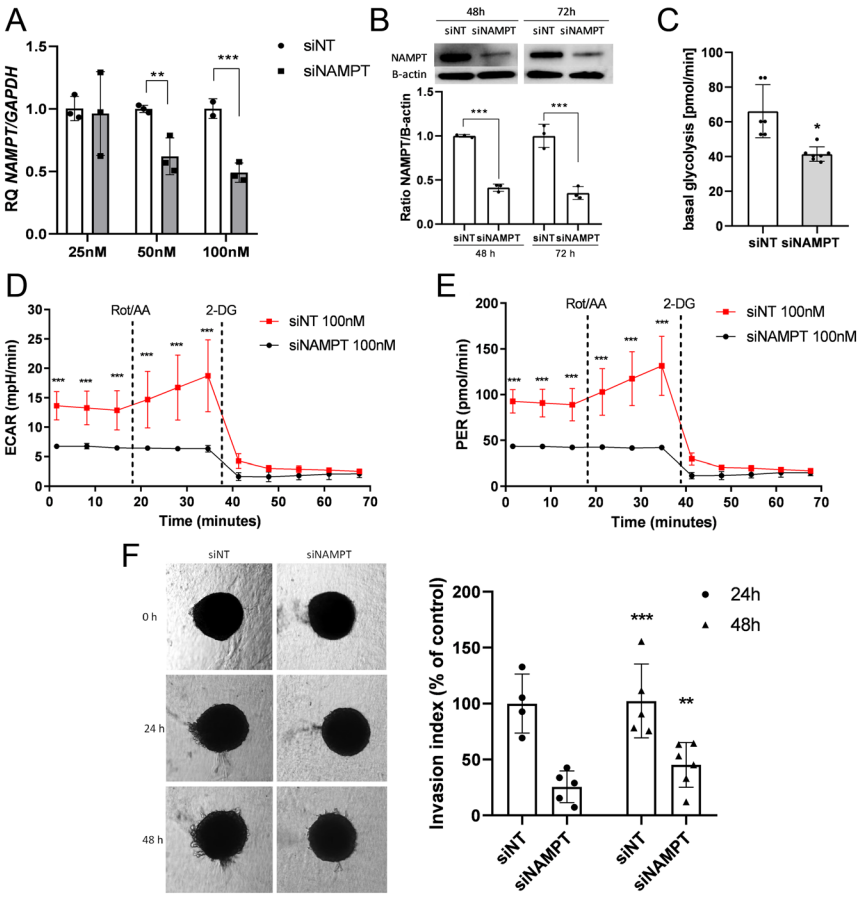


Figure 7 Effect of visfatin (NAMPT) silencing on glycolysis and invasive potential in KGN cells. Expression of NAMPT mRNA 24 h after transfection with nontargeting (siNT) or NAMPT-specific (siNAMPT) siRNA (A) and protein expression of NAMPT 48 h and 72 h after transfection with siNT or siNAMPT siRNA (B). Effects of NAMPT silencing on basal glycolysis (C), the ECAR (D), the PER (E), and invasive potential (F) in KGN cells. RQ, relative quantity. Data represent the mean ± S.E.M. of three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

of solid tumors and metastasis of breast cancers (Kim *et al.* 2010). Here, we show that visfatin increases the invasiveness of KGN cells but not that of SKOV-3 cells. Additionally, NAMPT silencing decreased the invasiveness of KGN cells. Furthermore, treatment of KGN cells with an inhibitor of visfatin (FK866) abolished the effect of visfatin on invasiveness. Consistent with these findings, two visfatin inhibitors, FK866 and GMX1778, show potent anticancer activity, as evidenced their negative effects on the growth of ovarian carcinomas (Tan *et al.* 2013, Zhang *et al.* 2018). Moreover, Nacarelli *et al.* (2020) revealed that FK866 in conjunction with platinum-based chemotherapy is a promising therapeutic strategy for ovarian cancer.

Interestingly, we showed that KGN cells were more invasive than SKOV-3 cells by examining the expression of genes encoding epithelial and mesenchymal proteins involved in EMT, an important facilitator of tumor

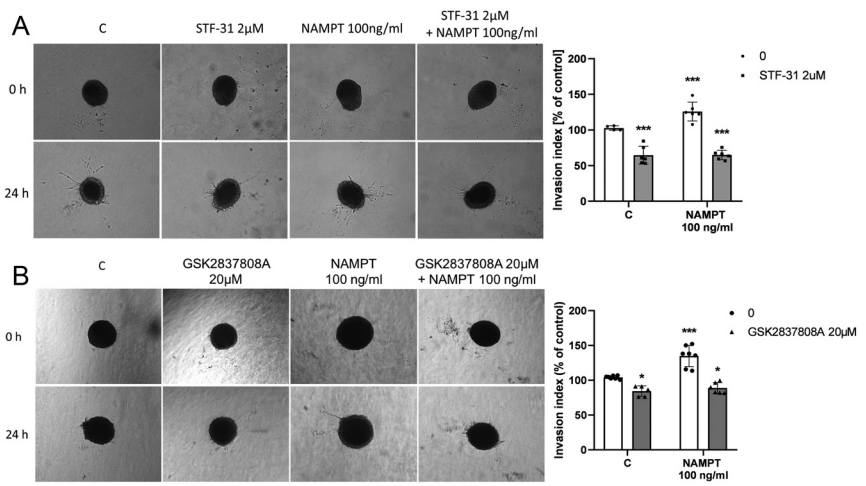


Figure 8 Effect of STF-31 (2 μM) (A) and GSK2837808A (20 μM) (B) on the invasiveness of KGN cell spheroids after treatment with visfatin (NAMPT) (100 ng/mL) for 24 h. Data represent the mean ± S.E.M. of three independent experiments. C, control. $^{*}P < 0.05$, $^{***}P < 0.001$.

cell invasiveness, in each of these tumor cell types. Specifically, the expression of epithelial genes, claudins, and E-cadherin was lower in KGN cells than in SKOV-3 cells, whereas that of N-cadherin was significantly higher. Studies show that high expression of N-cadherin increases the invasive capacity of cancer cells (Luo *et al.* 2018). Moreover, low E-cadherin expression and high N-cadherin expression are associated with ovarian cancer metastasis and peritoneal dissemination (Sawada *et al.* 2008). In the present study, we found 89-fold higher expression of MMP-2 in KGN cells than in SKOV-3 cells. Indeed, MMP-2 expression correlates positively with the invasiveness of ovarian GCT (Gogola-Mruk *et al.* 2021) and epithelial ovarian cancer (Zhang *et al.* 2006). These findings indicate that KGN cells have a more mesenchymal phenotype than SKOV-3 cells, which may explain the greater invasiveness of the former. Furthermore, we showed that visfatin increased the expression of MMP2, which is crucial for EMT and cancer invasiveness, and reduced that of the epithelial genes claudins 3 and 4 in AGCTs but not in epithelial ovarian cancer. The loss of the tight junction barrier facilitates metastasis, invasion, and motility of tumors (Martin & Jiang 2009). Similarly, visfatin also facilitates the migration of chondrosarcoma cells in mice through the upregulation of MMP-2 expression (Hung *et al.* 2021). The present findings are consistent with the previous observation that visfatin promotes the migration of a colorectal carcinoma cell line by inducing EMT (Yang *et al.* 2016). Thus, our findings imply that the effect of visfatin on KGN spheroid invasiveness is mediated via changes in the expression of genes and proteins that mediate EMT.

Previous studies have explored crosstalk between cancer metabolism and EMT, both of which are important for cancer metastasis (Thiery & Sleeman 2006). Glycolysis represents the initial stage in the breakdown of glucose following its uptake into cells by GLUTs. We found that the expression of GLUT1 mRNA was higher in KGN cells than in SKOV-3 cells. Importantly, GLUT1 is highly expressed in, and facilitates metastasis of, tumors; it is also associated with a poor prognosis of high-grade serous ovarian cancers (Pizzuti *et al.* 2018). GLUT1 plays an important role in the regulation of spheroid formation in a 3D culture model. Transfection with GLUT1 siRNA decreased the size of formed hepatocellular carcinoma spheroids (Amann *et al.* 2009). In contrast, upregulating GLUT1 increased the size of breast cancer in 3D culture (Singh *et al.* 2016). Gandham *et al.* (2015) reported that GLUT-1 was highly expressed in SKOV-3 spheroids. Furthermore, visfatin increases glucose uptake and GLUT1 protein expression in KGN cells but not in SKOV-3 cells. Similarly, visfatin treatment dose dependently induces rapid glucose uptake and increases the expression of GLUT1 protein in rat mesangial cells and human osteoblasts (Xie *et al.* 2007, Song *et al.* 2008). Thus, visfatin regulates the expression of GLUT1 and thereby affects glucose uptake in AGCTs. We also measured GLUT4 expression in the present study but found that visfatin does not affect GLUT4 protein expression in KGN cells. However, in skeletal muscle cells, visfatin increases the expression of GLUT4 and its translocation to the plasma membrane (Lee *et al.* 2015), which suggest that the expression of GLUT is tissue dependent.

The enzyme HK2, which catalyzes the first step of glycolysis, plays an important role in tumor invasiveness and is involved in the induction of EMT (Kang *et al.* 2019). Previous studies show that HK2 expression is high in cancer cells and that it correlates with epithelial ovarian cancer metastasis (Siu *et al.* 2019). We found that basal expression of *HK2* was significantly higher in KGN cells than in SKOV-3 cells and that visfatin increases HK2 activity in KGN cells without affecting the gene expression. The drastically suppressed expression of *HK2* in transgenic mice without significant suppression of HK activity has been observed (Ogawa *et al.* 2020). However, the discrepancy in the change of HK2 expression and activity remains unclear. At the end of this pathway, pyruvate is metabolized by the cyclic acid cycle, or by LDHA, to form lactate. In addition, we demonstrated that visfatin increases the expression and activity of LDHA in KGN cells. To the best of our knowledge, no study has characterized the direct effects of visfatin on the expression and activity of HK2 and LDHA in human ovarian cancer cells *in vivo* or *in vitro*. However, our results confirm indirectly the findings of Guo *et al.* (2021), who showed that the expression of LDHA mRNA and protein is lower in liver cancer cells (HepG2) treated with an NAMPT inhibitor (FK866).

A key player in cancer metabolic reprogramming is HIF1, which orchestrates the shift from glucose oxidation to aerobic glycolysis; this process is characteristic of cancer metabolism and is essential for invasiveness of tumor cells (Han *et al.* 2013). Here, we show for the first time that visfatin stimulates the expression of HIF-1 protein in KGN cells. Moreover, we show that basal and stimulated glycolysis in KGN cells is increased by visfatin and that silencing NAMPT expression significantly reduces basal glycolysis. The effect of visfatin on cancer metabolic reprogramming has also been shown in metastatic melanoma (Audrito *et al.* 2020). Furthermore, inhibition of visfatin reduces cellular NAD^+, glycolytic flux, mitochondrial function, and ATP levels in tumors (Tan *et al.* 2013). These findings indicate that visfatin regulates glucose metabolism in ovarian GCTs.

Finally, we demonstrated that pretreatment of KGN cells with an inhibitor of GLUT1 (STF-31) or LDHA (GSK2837808A) reduces their invasiveness and abrogates the effect of visfatin. These findings are consistent with those of Oh *et al.* (2017), who reported that knockdown of GLUT1 in breast cancer cells reduces their glucose uptake, migration, and invasiveness. Other studies have shown that upregulation of LDHA

is critical for the maintenance of glycolysis and the invasiveness of cancer cells (Baumann *et al.* 2009). Our study is the first to evaluate the relationship between the effects of visfatin on glucose metabolism and invasiveness of ovarian GCT. The data indicate that glucose availability is essential for AGCT spheroid invasiveness. Furthermore, our research highlights the role of visfatin in regulating the invasiveness of ovarian GCT spheroids by modulating the glycolysis process. A visfatin receptor has still not been identified. However, visfatin receptor-dependent action has been suggested through, among others, the insulin receptor (Kang *et al.* 2010). Studies by Romacho *et al.* 2020 also indicate the involvement of Toll-like receptor-4 and nod-like-receptor-protein-3 inflammasome. It can be suspected that the insulin receptor, expressed in KGN and SKOV-3 (Beauchamp *et al.* 2010, Rice *et al.* 2011), is involved in a potential mechanism by which visfatin affects invasion and glucose metabolism in ovarian GCT. However, this has not been clarified, and further research is required.

In summary, visfatin increases the invasiveness of adult ovarian GCTs by playing an important role in the modulation of glucose metabolism in AGCT cells. These data imply that visfatin, its inhibitor or *NAMPT* silencing may be a potential candidate for the diagnosis and treatment of ovarian GCT.

Declaration of interest

The authors declare no conflicts of interest.

Funding

This work was supported by the National Science Centre (NCN), Poland (Preludium 17; grant number: 2019/33/N/NZ5/00471). The cost of Open Access publication was covered by the Society for Biology of Reproduction in Poland.

Author contribution statement

JG and AP, designed and directed the project; JG, WM, and KK performed the experiments; JG and AP were involved in planning and supervised the work, JG processed the experimental data, performed the analysis, drafted the manuscript, and designed the figures.

Acknowledgements

The authors thank Professors Toshihiko Yanase and Yoshihiro Nishi for the KGN cells (generously provided by the Riken Cell Bank, Koyadai, Japan).

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Received 29 November 2022
Accepted 7 March 2023
Available online 7 March 2023
Version of Record published 30 March 2023